Elucidating the Function and Cell-Specific Interactions of P2X7 Receptor Variants Linked to Mental Disorders

Poulsen, Mette Homann; Fang, Jamie; Pless, Stephan A.

DOI:
10.1016/j.bpj.2018.11.624

Publication date:
2019

Document version
Publisher's PDF, also known as Version of record

affinity and specificity for each NBS, the interactions between the various NBSs, and the mechanism whereby nucleotide binding events are transmitted to the channel pore are largely unknown. To fully understand the nucleotide effects on K_{ATP}, we have labelled each NBS (one at a time) with the fluorescent, non-canonical amino acid ANAP and measured nucleotide binding at each site using FRET between ANAP and trinitrophenyl (TNP) nucleotide derivatives in unroofed membrane fragments. This has allowed us to dissect nucleotide binding to each site, evaluate mechanistically mutations expected to affect nucleotide binding (e.g. SUR1-K1384A, Kir6.2-G334D) or channel gating (e.g. Kir6.2-C166S), and investigate the action of clinically important drugs that inhibit or potentiate K_{ATP} (sulfonylureas and K^+ channel openers). We have also combined this method with patch-clamp electrophysiology (either in separate experiments or simultaneously with patch-clamp fluorometry) to determine the functional consequences of binding at each NBS. This method will not only yield insights into K_{ATP} channel activity, but is readily extended to other channels, ABC transporters, and virtually any protein with a suitable fluorescent ligand.

543-Pos

Single Molecule FRET Reveals Lipid Induced Conformational Changes in Cytoplasmic Domain of Kir2.1

Joshua B. Brettmann[1], Sun Joo Lee[1], Shizhen Wang[2], Colin G. Nichols[1].
[1]Dept Cell Biol, Washington Univ St Louis, St Louis, MO, USA, [2]School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO, USA.

Anionic phospholipids are common regulators of ion channel activity, including Kir2.1, an inward rectifying potassium channel crucial for setting and maintaining resting membrane potential in many tissues, which is activated specifically by PIP2 and phosphatidyl glycerol (PG). Crystal structures indicate that a simple upward translocation of the cytoplasmic Kir domain is induced by PIP2 and PG binding. However, recent computational and single-molecule FRET experiments show more complex intra-domain movements and rotation in the Kir domain of prokaryotic KirBac1.1 upon PIP2 binding. Single molecule FRET measurements on eukaryotic human Kir2.1 indicate similar complex motions that are not detected in static crystal structures. Our measurements indicate dynamic conformational 'breathing' throughout the Kir domain, suggesting greater flexibility in the intracellular domain of ion channels than may be apparent in structural studies. Specifically, our data indicate a PIP2 dependent shifts in average single molecule FRET (smFRET) distributions, with outward motions seen prior to the slide helix (residue 59) and an inward motion seen at the base (residues 286 and 288) consistent with a twisting motion of the Kir domain that was previously shown to be associated with opening in KirBac1.1. In the absence of PIP2, smFRET signals are very dynamic with relatively large (>20A) motions at specific residues. PIP2 stabilizes the conformational dynamics of the Kir domain, with less transitions between structural states, and reduction in the occurrence of large conformational transitions.

544-Pos

Multiple Nucleotide-Dependent Conformations of a Mycobacterial RCK Domain

Alexandre G. Vouga[1], Katia K. Matychak[1], Michael E. Rockman[1], Lisandra Flores[2], Sebastian Brauchi[2], **Brad S. Rothberg**[1].
[1]Dept Med Genetics Mol Biochem, Temple Univ Sch Med, Philadelphia, PA, USA, [2]Dept Physiology, Universidad Austral de Chile, Valdivia, Chile.

Potassium (K) channels play a critical role in bacterial electrolyte homeostasis and control of membrane potential, and are thus important in bacterial physiology. Here we present X-ray crystal structures of the ligand-binding RCK domain from the nucleotide-sensing *Mycobacterium intracellulare* K channel, in complex with ATP, ADP, and AMP. In this RCK domain, ATP is coordinated by a hydrogen bond between N6 (on the adenine base) and the side chain of Asp-200, a pair of hydrogen bonds between O2' and O3' (on the ribose sugar) and the two side chain oxygens on of Asp-180. In addition, an intersubunit salt-bridge is formed between the gamma phosphate of ATP and Arg-249, and an intersubunit H-bond is also formed between the gamma phosphate and Ser-260. The ATP-bound RCK complex forms a four-fold symmetric ring, in which each ATP-bound subunit is in an identical conformation. Co-crystallization with either ADP or ATP, however, yields a two-fold symmetric ring in which the Ser-260 H-bond is absent in alternating subunits. To determine the energetic roles of these residues in nucleotide coordination, we estimated ATP affinities of WT and mutant RCK domains using isothermal titration calorimetry. We observed that the wild-type RCK domain bound ATP with a Kd of 4.1 +/- 0.2 μM, whereas mutation of Arg-249 to disrupt the salt-bridge (R249A) yielded a Kd of approximately 270 μM. This corresponded to a free energy change of approximately 2.5 kcal/mole per binding site. In the structure, the R249A mutant binds ATP with decreased phosphate coordination, and was not stabilized in the four-fold symmetric conformation observed with the wild-type domain. These data indicate that the mycobacterial K channel can distinguish among ATP, ADP, and AMP to sense the cell's metabolic state and potentially transduce nucleotide binding to control K conduction.

545-Pos

Rck Domains can Assemble as Hetero-Octamers and Control Different Ligand-Gated Channels

Rita Rocha, Celso Teixeira Duarte, Joao M.P. Jorge, Joao H. Morais Cabral.
I3S - Instituto de Investigacao e Inovacao em Saude, Porto, Portugal.

Potassium (K^+) uptake is essential in all living cells. In non-animal organisms K^+ transport assumes a major role in adaptation to osmotic stress and high salinity. RCK (regulating conductance of K^+) domains are widespread in nature, where they regulate the activity of eukaryotic potassium channels and prokaryotic K^+ channels and transporters.

Bacteria in particular express different families of K^+ transporters and channels, some of which are regulated by RCK domains, that allow growth under different K+ limiting concentrations. Two or more different K^+ uptake systems can be present in a bacterial cell and, some species contain more than one member of the same family. We are exploring the biological role of this functional redundancy in *Bacillus subtilis* Ktr K^+ transporters, which are regulated by RCK domains. *B. subtilis* genome codes for two membrane proteins (KtrB and KtrD) with structural similarities to K+ channels and two cytosolic regulatory RCK domain proteins (KtrA and KtrC). Since KtrA and KtrB form an operon, while KtrC and KtrD are located in different loci of the chromosome, the KtrAB and KtrCD complexes have been proposed as two independent Ktr transporters. We have observed complex formation between the different subunits by size-exclusion chromatography and functional complementation assay. We observed that the two RCK domain proteins are able to interact and activate both KtrB and KtrD; moreover, when incubated together, KtrA and KtrC can also assemble as mixed octameric rings. These results unveil a novel feature of RCK domains with implications on the mechanisms of regulation of K^+ transport.

546-Pos

Characterizing $P2X_2$ Mutants Associated with Progressive Sensorineural Hearing Loss (DFNA41)

Benjamin I. George, Mufeng Li, Kenton J. Swartz.
NINDS NIH, Bethesda, MD, USA.

P2X receptors are trimeric ion channels that open in response to extracellular ATP. Each subunit is comprised of intracellular N- and C-termini, a large extracellular domain containing the ATP binding site and two transmembrane (TM) helices that form a pore that is permeable to cations. $P2X_2$ receptors are hypothesized to play a role in cochlear adaptation to elevated sound levels and protection from overstimulation. Whole-exome sequencing and linkage analysis of individuals with dominantly inherited progressive sensorineural hearing loss (DFNA41) revealed three different mutations of human $P2X_2$ ($hP2X_2$) receptor: V60L, D201Y, and G353R. To investigate the effects of mutations, recombinant $hP2X_2$ receptors were expressed in HEK 293 cells and macroscopic currents were measured with the whole-cell patch clamp technique. We find that the G353R mutation lowers the apparent affinity for activation by ATP and produces a pronounced inward rectification, with large inward currents being observed only below −80 mV. In contrast, expression of the V60L mutation produces measurable constitutive currents with little rectification. To verify that the constitutive currents observed with V60L arise from the mutant receptor, we inserted a Cys into the pore-lining TM2 helix and observed robust and irreversible inhibition by thiol-reactive methanethiosulfonates (MTS). Interestingly, larger MTS reagents reacted more slowly with the V60L construct compared to control, suggesting that the mutation produces a structural alteration in the pore. Finally, the D201Y mutant channel does not respond to ATP and appears to be non-functional. Taken together, our results suggest that all three mutations greatly diminish or ablate ATP-activated currents at physiological voltages, and that the V60L also produces constitutively active channels. It will be interesting to further explore how these perturbations give rise to severe progression of hearing loss.

547-Pos

Elucidating the Function and Cell-Specific Interactions of P2X7 Receptor Variants Linked to Mental Disorders

Mette Homann Poulsen, Jamie Fang, Stephan A. Pless.
Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen, Denmark.

With a quarter of the world's population affected by neurological or mental disorders at some stage of life, there is great demand for understanding the

molecular basis of these disorders. In the CNS, activation of microglial P2X7 receptors (P2X7Rs) triggers neuroinflammation, which is intricately linked to some mental disorders. P2X7Rs belong to the family of adenosine triphosphate (ATP)-gated ion channels, which are trimeric receptor assemblies. Interestingly, the P2X7R displays an unusually high number of single nucleotide polymorphisms (SNPs) across the population, resulting in numerous SNP-containing receptor variants, some of which are linked to diseases, such as mental disorders. Notably, the functional and pharmacological consequences of P2X7R SNPs remain unexplored. To gain a comprehensive understanding of the function of pathophysiologically relevant P2X7R SNP variants, we study halotypes of the human P2X7R found in patients suffering from mental disorders. The functional and pharmacological characterization of these halotypes is performed both in human microglia and HEK cells, using patch-clamp and high-throughput fluorescence assays. Additionally, both wild type P2X7Rs and SNP variants have been suggested to play diverse and sometimes contradictory roles in different cell types. We hypothesize that this differential role is caused by cell-specific protein-protein interactions (PPIs) between the P2X7R N- and/or C-terminal domains and intracellular proteins, which we aim to identify and characterize using mass spectrometry. Together, this starts to disentangle the molecular basis of disease-linked microglial P2X7R variants, leading to a better understanding of mental disorders.

548-Pos

Stomatin Dependent Regulation of the Acid Sensing Ion Channels

Robert C. Klipp, John Bankston.
Physiology and Biophysics, University of Colorado Anschutz Medical Campus, Aurora, CO, USA.

In the peripheral and central nervous systems, Acid Sensing Ion Channels (ASICs) are the primary mediators of enhanced neuronal activity due to extracellular acidification. Despite ASICs importance in regulating pathophysiological conditions of tissue acidosis, many of the molecular mechanisms underlying ASIC function, such as its incorporation into higher order ion channel complexes, remain poorly elucidated. Stomatin, a member of the SPFH family of integral membrane proteins has previously been shown to complex with ASIC isoforms 1, 2 and 3, while having differential regulation of all 3. Stomatin shows a near complete inhibition of ASIC3 function when recombinantly expressed in mammalian cells. However, in DRG sensory neurons, where Stomatin and ASIC3 are known to interact, ASIC3 activates to moderate acidifications. Here, we present our preliminary findings investigating several aspects of the molecular mechanisms underlying Stomatin's regulation of ASIC3. First, we seek to localize both regulatory and non-regulatory Stomatin binding sites, creating a systematic series of truncated, chimeric, and point mutant channels. Mutant channels are recombinantly expressed into mammalian cells and patch clamp electrophysiology is utilized to examine channel function in the presence and absence of Stomatin. We use FRET to pair mutant channel function with Stomatin binding using the respective fluorescently labelled constructs. Finally, we investigate the role that domains on Stomatin play in the dynamic regulation of ASIC3.

549-Pos

Indirect Determinants of Ion Selectivity in Acid-Sensing Ion Channels and Epithelial Sodium Channels

Zeshan P. Sheikh[1], Timothy Lynagh[2], Anders S. Kristensen[1], Stephan A. Pless[1].
[1]Drug Design and Pharmacology, Univ Copenhagen, Copenhagen, Denmark, [2]Univ Copenhagen, Copenhagen, Denmark.

Members of the ENaC/DEG superfamily of ion channels are Na^+-selective ion channels with a common trimeric architecture. Each of the three subunits has two transmembrane helices (M1 and M2), of which M2 lines the pore. Members of this family include the acid-sensing ion channels (ASICs), formed by identical or homologous subunits that mediate excitatory Na^+ currents in the nervous system (relative Na^+/K^+ permeability of approx. 10/1). The epithelial sodium channels (ENaCs) are obligate heterotrimers and display a 10-fold higher Na^+ selectivity than ASICs. The most recent findings indicate that this discrepancy might be due to different selectivity filter (SF) locations; while the ENaC SF is likely formed by the conserved G/S-X-S motif in the center of the pore, we have recently shown the mouse ASIC1a SF to be composed of two acidic side chains in the lower part of M2, namely E18' and D21'. In order to elucidate if other parts of the channel contribute to the stark differences in Na^+ selectivity between ASICs and ENaCs, we used conventional and non-canonical amino acid substitutions to probe the contribution of M1 residues to ion selectivity. Our results show that aromatic residues in ASIC M1 are important for ion selectivity. ENaC contains additional aromatic residues in M1. We hypothesize that these aromatics are similarly important for ion selectivity and that pore diameter plays an important role in both channels. Furthermore, the intracellular domains have previously been suggested to contribute to ion selectivity. Using a novel split intein-based approach we fuse partial ASIC1a constructs with recombinant or synthetic peptides corresponding to the N- or C-terminus of the full length protein. This enables us to introduce non-canonical amino acid substitutions, including post-translational modifications, into these less-studied regions of the channel.

550-Pos

Molecular Basis for Ion Selectivity in Heteromeric Acid-Sensing Ion Channels

Zeshan P. Sheikh[1], Timothy Lynagh[2], Emelie Flood[3], Celine Boiteux[4], Toby W. Allen[5], Stephan A. Pless[1].
[1]Drug Design and Pharmacology, Univ Copenhagen, Copenhagen, Denmark, [2]Univ Copenhagen, Copenhagen, Denmark, [3]RMIT Univ, Brunswick, Australia, [4]Hlth Innovat Res Inst, RMIT Univ, Melbourne, Australia, [5]Sch of Sci, RMIT Univ, Melbourne, Australia.

Acid-sensing ion channels (ASICs) occur throughout the nervous system and open in response to proton binding. Most ASICs are ~10-fold selective for Na^+ over K^+, and we recently showed that in homomeric ASIC1a channels, preferential Na^+ conduction is primarily controlled by glutamate (E18') and aspartate (D21') side chains at the intracellular end of the pore. Additionally, two leucine residues (L7' and L14') and a constriction termed the GAS belt (G10'-S12') also contribute to ion selectivity. However, it remained unclear if this mechanism extends to other ASIC isoforms, including heteromeric channels. Here, we investigated the molecular determinants of ion selectivity in homomeric ASIC2a and heteromeric ASIC1a/ASIC2a channels, using site-directed mutagenesis, electrophysiology and molecular dynamics simulations. In contrast to ASIC1a, L7'A mutation had no effect on ion selectivity in ASIC2a. L14' and, especially E18' mutations, however, substantially decreased selectivity of ASIC2a, while G10' and S12' mutations rendered channels non-functional, as in ASIC1a. Coexpression of mutant ASIC2a with WT ASIC1a (and vice versa) led to functional heteromeric channels containing 1-2 mutated subunits. In these heteromers, E18' mutations had stronger effects on ion selectivity than any other position tested here. This was consistent with simulations, showing favorable interactions of E18' side chains with Na^+ ions in both homomeric ASIC2a and heteromeric ASIC1a/ASIC2a channels. Furthermore, simulations provided an explanation for the reduced role of L7' in ASIC2a channels, revealing a distinct free energy profile above the central GAS region, due to attractive interactions with carboxylate-containing residues at the upper end of the pore, rendering the L7' site non-rate determining. These results suggest a more pronounced role of the external mouth of the pore and the GAS belt in ASIC2a compared to ASIC1a, but confirm that E18' is crucial to ion selectivity in various ASIC isoforms.